Yamamoto, 1996b), a rifampicin-resistant mutant derived from strain W3110, and *Serratia marcescens* CU237rif, a rifampicin-resistant mutant derived from the antibiotic susceptible clinical isolate CU237, were used as the recipient strains. *E. coli* ML1410 (O'Hara, 1993) carrying plasmid RP1 (Ingram *et al.*, 1972; Kono *et al.*, 1980) was used as a positive control for transconjugation of drug resistance.

Media and antibiotics

Nutrient broth (Difco Laboratories, Inc., Detroit, Michigan, U.S.A.) was used as a liquid medium and nutrient agar was used as a solid medium. Reduced glutathione was purchased from Wako Pure Chemical Industries Ltd., Tokyo, Japan. The antibiotics investigated were sodium fosfomycin (FOM), tetracycline (TC), carbenicillin (CBPC), chloramphenicol (CP), streptomycin sulphate (SM), kanamycin (KM), gentamicin (GM), nalidixic acid (NA), norfloxacin (NFLX), neomycin sulphate (NEO), rifampicin (RFP) (Sigma Chemical Co., St Louis, Michigan, U.S.A.), and sulphonamide (SA) (sodium sulphisomidine; Dainippon Pharmaceutical Co., Ltd, Osaka, Japan).

Identification of bacteria

The identification of clinical isolates was performed using a Vitek autoanalyser (BioMérieux Vitek, Inc., U.S.A.) and was confirmed by tests (Table 1) with: 2,4,4'-trichloro-2'-hydroxydiphenylether (DP3), glucose (OFG), L-tryptophan (GC), acetamide (ACE), aesculin (ESC), indoxyl-β-D-glycoside (PLI), urea (URE), sodium citrate (CIT), sodium malonate (MAL), tryptophan (TDA), polymyxin-sulphate (PXB), lactose (LAC/TLA), maltose (MLT), D-mannitol (MAN), D-xylose (XYL), D-raffinose (RAF), D-sorbitol (SOR), sucrose (SUC), inositol (INO), adonitol (ADO), *p*-coumaric acid (COU), sodium thiosulphate (H_2S), *O*-nitrophenyl-β-D-galacto-pyranoside (ONG), D-rhamnose (RHA), L(+)-arabinose (ARA), glucose (GLU), L-arginine (ARG), L-lysine (LYS), L-ornithine (ORN), and decarboxylation (OXI). The properties of strain CU264 were compared with those of *S. marcescens* CU231.

Antibiotic susceptibility testing

The concentrations of antibiotics in the test plates were as follows: 100 μg/ml FOM, 25 μg/ml TC , 500 μg/ml CBPC, 25 μg/ml CP, 20 μg/ml SM, 50 μg/ml KM, 25 μg/ml GM, 25 μg/ml NA, 25 μg/ml NFLX, 50 μg/ml NEO, and 25 μg/ml RFP. Resistance to 400 μg/ml SA was tested using Müeller-Hinton agar plate. The determination of minimal inhibitory concentrations (MICs) of FOM were performed by the agar double-dilution method. In this technique 1,000-fold dilutions of overnight cultures in nutrient broth were inoculated on nutrient agar plates containing serial twofold dilutions of FOM. The MICs were determined after incubation at 37°C for 18 h.

Conjugal transfer

Modifications to the conjugal transfer method described by O'Hara (1993) and Kono and O'Hara (1975) were as follows. For the broth method, each donor (0.1 ml) and each recipient (0.1 ml) of the exponentially growing cultures were mixed with 0.8 ml of nutrient medium and incubated overnight at 37°C. Mixed cultures (0.01 ml) were spread on selective plates.

For the filter mating method, exponentially growing cultures of each donor (2 ml) and recipient (2 ml) were mixed, and the mixed culture was filtered through a membrane filter (type HA, 0.45 µm, Millipore Corporation, Bedford, Massachusetts, U.S.A.). The cells were cultured overnight on nutrient agar at 37°C and were suspended in 4 ml of 0.9% NaCl. Then 0.01 ml of the suspension was spread onto a nutrient agar plate containing the selective agents. After incubation overnight at 37°C, the colonies on the plates were counted and the transfer frequency of drug resistance was determined.

Results and discussion

Strain CU264 was isolated from samples from patients with chronic urinary tract infection. The colonies were tested for Gram-staining and the strain was identified as a Gram-negative rod bacterium.

In the process of identifying the bacterial strain CU231 which was used as a standard, properties which were 99% consistent with *S. marcescens* were evident. In contrast with strain CU231, it was found that strain CU264 was positive for MAL, XYL, RAF, ONP, RHA and ARA, and negative for DP3, PXB, ADO, COU, LYS and ORN (Table 1). Analysing the result using Vitek's chart according to Balows *et al.* (1994), the confidence level of the identification of strain CU264 as *R. aquatilis* was 90%, and for *Enterobacter intermedius* was 9%. Based on these results, it was concluded that strain CU264 was *R. aquatilis*.

Resistance to antibiotics of strain CU264 was examined (Table 2). As a result, it was revealed that strain CU264 was resistant to FOM only. The MIC for FOM was >1,600 µg/ml, showing that strain CU264 was a high FOM-resistant bacterium. Since no other *R. aquatilis* clinical isolate has been reported, except for strain CU264 in Japan, a comparison with strains of the same species is not possible at this stage. Izard *et al.* (1985) did not indicate that *R. aquatilis* had an essential resistance to any particular antibiotic, suggesting that this FOM resistance was mediated by an exogenous gene.

Furthermore, it appeared that the transmission of FOM resistance in *R. aquatilis* to *E. coli* W3110rif had not occurred either by broth

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Table 1 Properties of *R. aquatilis* CU264 and *S. marcescens* CU231 tested by Vitek system

Test used	Strains: CU264	CU231
DP3	−	+
OFG	+	+
GC	+	+
ACE	−	−
ESC	+	+
PLI	+	+
URE	−	−
CIT	+	+
MAL	+	−
TDA	−	−
PXB	−	+
LAC/TLA	−	−
MLT	+	+
MAN	+	+
XYL	+	−
RAF	+	−
SOR	+	+
SUC	+	+
INO	−	−
ADO	−	+
COU	−	+
H_2S	−	−
ONG	+	−
RHA	+	−
ARA	+	−
GLU	+	+
ARG	−	−
LYS	−	+
ORN	−	+
OXI	−	−

Table 2 Antibiotic resistance of strains used in this work

Strain	Phenotype of antibiotic resistance*:						
R. aquatilis CU264	FOM						
S. marcescens CU231		TC				NA	RFP
E. coli ML1410/RP1		TC	CBPC	KM	NEO	NA	
E. coli W3110rif							RFP

* Concentrations of antibiotics in the plates were: FOM, 100 µg/ml; TC, 25 µg/ml; CBPC, 500 µg/ml; CP, 25 µg/ml; SM, 20 µg/ml; KM, 50 µg/ml; GM, 25 µg/ml; SA, 400 µg/ml; NA, 25 µg/ml; NEO, 50 µg/ml; NFLX, 25 µg/ml; and RFP, 25 µg/ml.

mating or by membrane filter mating. The transmission of plasmid RP1 carried by *E. coli* ML1410 was successful when used as a standard under the same conditions (Table 3). In an attempt to transmit the FOM resistance to *S. marcescens* CU237rif, in which the biochemical properties were similar to *R. aquatilis*, a 4 x 10^{-6} transmission frequency of FOM resistance was observed by the membrane filter mating method although the frequency by the broth mating method was <10^{-6}.

Table 3 Transfer frequencies of FOM resistance to *S. marcescens* CU237rif and to *E. coli* W3110rif

Donor	Recipient	Transfer frequency: Selected markers*	Broth mating (CFU/ml)	Filter mating (CFU/ml)
R. aquatilis CU264	E. coli W3110rif	FOM/RFP	<1 x 10^{-6}	<1 x 10^{-6}
	S. marcescens CU237rif	FOM/RFP	<1 x 10^{-6}	4 x 10^{-6}
E. coli ML1410/RP1	E. coli W3110rif	TC/RFP	3 x 10^{-5}	1 x 10^{-3}

* TC, 25 µg/ml; RFP, 25 µg/ml; FOM, 25 µg/ml.
CFU, colony-forming units.

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In Europe, a FOM resistance element was present in the R plasmid with a multidrug resistance element and was easily transmitted by mixed culture with other enterobacteria in liquid culture medium (Mendoza et al., 1980; Suarez and Mendoza, 1991). It is known that the genes fosA (Villar et al., 1986) in FOM-resistant Serratia marcescens and fosB (Zilhao and Courvalin, 1990) in Staphylococcus epidermidis encode the GSH addition enzymes (Suarez and Mendoza, 1991). Recently, the genes fosC (Garcia et al., 1995) in FOM-producing Pseudomonas syringae, and fomAB (Kuzuyama et al., 1996) in Streptomyces wedmorensis encoding the FOM phosphorylation enzyme have been reported. To our knowledge, there are only a few FOM-resistant fosA-positive bacteria in Europe. It has also been reported that a change in membrane permeability caused alterations in FOM incorporation in one strain (Arca et al., 1997).

On the other hand, in Japan, FOM-resistant bacteria are rare (O'Hara et al., 1988; Tsuboi et al., 1998) and Klebsiella pneumoniae is resistance-transmissible against FOM with a low frequency (O'Hara, 1993). It is suggested that the effect of FOM in combination with other antibiotics is the reason that there are only a few FOM-resistant bacteria found in Japan (O'Hara and Hashimoto, 1996a; O'Hara et al., 1997b).

In the present work, we describe a strain which is highly resistant to FOM. It was identified as R. aquatilis, which has rarely been isolated clinically. However, the resistance was not transmitted to E. coli but was transmitted to S. marcescens. Therefore, we are particularly interested in the mechanism by which FOM resistance is acquired and how the gene encoding FOM resistance in R. aquatilis, as described in this report, was initially acquired. The analysis of this gene and the resistance mechanism is an ongoing task which is currently being undertaken in our laboratories.

Acknowledgement

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喀痰中 β-ラクタマーゼ活性の測定方法の確立に関する検討

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喀痰中β－ラクタマーゼ活性の測定方法の確立に関する検討

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喀痰から直接β－ラクタマーゼを検出することを目的に，*Pseudomonas aeruginosa* が分離された喀痰を対象とした β－ラクタマーゼ活性の測定方法を確立した。喀痰からの抽出操作は30分以内に終了した。*P. aeruginosa* が 10^6cfu/ml 存在する喀痰からの抽出液を 100μM のニトロセフィン溶液に添加して5分間37℃で経時的に吸光度を測定したところ，きわめて良好な直線関係を得ることができた。一方，10^6cfu/ml の *Enterococcus faecalis* が存在する検体からはβ－ラクタマーゼ活性は認められなかった。次に検出された菌量が異なる複数の検体を用いて検出限界に関する検討を行った。その結果，*P. aeruginosa* が 10^5cfu/ml 以上の菌量が存在する検体からはβ－ラクタマーゼ活性が検出された。また，ペニシリナーゼを産生する *Escherichia coli* が 10^7cfu/ml 存在する喀痰の K_m 値は 127μM と大きな値を示し，セファロスロリナーゼを産生する *P. aeruginosa* の値とは大きく異なった。以上の結果から，今回検討した方法は迅速，簡便かつ高感度で検体からβ－ラクタマーゼを検出することが可能な方法であった。

Key words: sputum, β–lactamase activity, nitrocefin, UV method

臨床では感染症の原因菌が同定される以前に経験的な抗菌薬の投与がなされる場合が多いのが現実である。感染症の原因菌と考えられる微生物の耐性因子を検体から直接検出することが可能になれば，菌の分離，同定あるいは薬剤感受性検査の結果が出る以前に，より的確な抗菌薬の投与が可能になると考えられる。これまでにも喀痰からのβ－ラクタマーゼ活性の測定に関しては，高速液体クロマトクラフィーを用いる方法が報告されている[1]が，操作が煩雑なことと結果が出るまでに時間を要することなどの理由から一般的な方法とはなっていない。

今回は検体から直接，簡便かつ迅速にβ－ラクタマーゼを酵素学的方法で検出することを目的として，現在最も高感度なβ－ラクタマーゼ検出試薬として知られているニトロセフィンを基質として用いる検出方法の確立を試みた。

I. 材料と方法

1. 対象とした喀痰

呼吸器疾患の患者由来の喀痰で *Pseudomonas aeruginosa* が分離された9検体を検討に用いた。さらに，典型的なペニシリナーゼを産生する *Escherichia coli* が分離された喀痰も使用した。なお，喀痰から分離された菌種はVitek（日本ビオメリュー，東京）を用いて同定した。

2. 喀痰からのβ－ラクタマーゼの抽出方法

喀痰 200μl を 1.5 ml のマイクロチューブに移し，1/15 M リン酸緩衝液（pH 7.0）200μl を加えてホモジナイザーでホモジネート後，$100,000\times$G，4℃ にて15分間超遠心機を用いて遠心分離し，その上清を酵素液とし

た（Fig. 1）。

3. 分離された菌体からのβ－ラクタマーゼの抽出方法

各菌株を L-broth で一夜培養した5 ml の菌液を3,500 rpm，4℃，15分間遠心分離して集菌した。その沈査を 50 mM のリン酸緩衝液（pH 7.4）で洗浄し，100μl の同緩衝液に再浮遊した後凍結融解を5回繰り返した。その後，$100,000\times$G，4℃，30分間の超遠心分離を行い，得られた上清を粗酵素液とした（Fig. 2）。

4. β－ラクタマーゼ活性の測定方法

酵素反応は Beckman 自記吸光光度計 DU 640（恒温槽付き）を用いて，基質のニトロセフィン溶液3 ml に試料酵素液 50μl を添加して，測定波長 482 nm，反応温度37℃ の条件[2]で UV 法[3]により行った（Table 1）。

実験にさきだち 100μM の濃度のニトロセフィン溶液に *P. aeruginosa* が 10^6cfu/ml 検出された喀痰からの抽出液，*Enterococcus faecalis* が 10^6cfu/ml 検出された喀痰からの抽出液を 50μl 添加して5分間のタイムスキャンを実施し，酵素との反応性を確認した。

基質のニトロセフィンは 10μM，25μM および 100μM の濃度の溶液を使用した。酵素活性の算出は，反応開始後1分間に生じる OD 値の変化をもとに次式から行った。Activitiy=y/1.59×0.3×v（v=3.05/0.05×2, y=ΔOD/min）さらに，酵素学的パラメータは，Beckman 自記吸光光度計 DU 640 付属の解析ソフトにより，Michaelis–Menten plot から算出した[4]。なお，実験で得られた値は，Lineweaver–Burk plot，Eadie–Hofstee

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plot, Hanes-Woolf plot の各プロットも同時に行い, 実験値の信頼性を確認した[4]。

5. 薬剤感受性試験

薬剤感受性試験は日本化学療法学会が定めた微量液体希釈法に準じて測定した。力価が明らかな piperacillin (富山科学工業株式会社, 東京), imipenem (萬有製薬株式会社, 東京), cefsulodin (武田薬品工業株式会社, 大阪), cefoperazone (ファイザー製薬株式会社, 東京), ceftazidime (日本グラクソ株式会社, 東京), cefepime (ブリストル・マイヤーズ・スクイブ株式会社, 東京)および aztreonam (エーザイ株式会社, 東京) の各抗菌薬を対象薬剤とした。なお, 培地は Muller-Hinton broth (Difco, USA) を使用し, 接種菌量は約 5×10^5 cfu/ml となるように設定した。

II. 結　　　果

1. ニトロセフィンと喀痰からの抽出液との反応性

P. aeruginosa が 10^6 cfu/ml 検出された喀痰からの抽出液 50 μl を用い, ニトロセフィン溶液と 5 分間, 30 秒毎に吸光度を測定しながら反応させた場合, きわめて良好な直線関係が得られた。同様に E. faecalis が喀痰から抽出された溶液の β-ラクタマーゼ活性 10^6 cfu/ml 検出された喀痰からの抽出液を用いて 5 分間, 経時的に吸光度を測定したが吸光度の変化は認められなかった (Fig. 3)。

2. 喀痰中 β-ラクタマーゼの活性測定

喀痰中からの抽出操作は 30 分以内に終了した。今回は P. aeruginosa が $10^5 \sim 10^7$ cfu/ml 分離された喀痰を対象に検討を加えた。P. aeruginosa が分離された喀痰からは 10^5 cfu/ml の菌量でも十分に β-ラクタマーゼ活性の測定が可能であり, 喀痰中に存在する β-ラクタマ

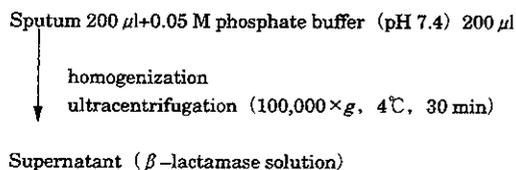Sputum 200 μl+0.05 M phosphate buffer (pH 7.4) 200 μl

homogenization
ultracentrifugation (100,000×*g*, 4℃, 30 min)

Supernatant (β-lactamase solution)

Fig. 1. Preparation of β-lactamase sample from sputum.

Strains cultured in L-broth
5 ml overnight

centrifugal collection of bacteria (3,500 rpm, 4℃, 15 min)
washing with 0.05 M phosphate buffer (pH 7.4) 1 ml
and re-suspension in the same buffer (100 μl)
freeze-defreeze, 5 times
ultracentrifugation (100,000×*g*, 4℃, 30 min)

Supernatant (β-lactamase solution)

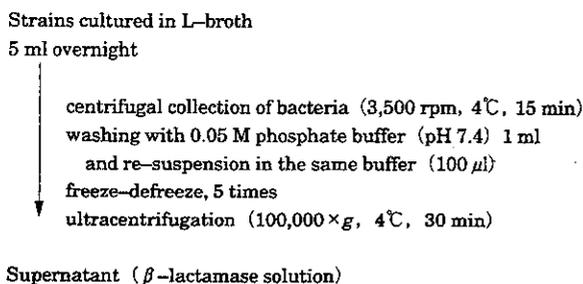**Fig. 2.** Preparation of β-lactamase sample from isolated strains.

ーゼの酵素学的パラメータを Michaelis-Menten plot から算出することが可能であった (Table 2)。さらに, そのデータを Lineweaver-Burk plot, Eadie-Hofstee plot, Hanes-Woolf plot の各 plot でも検討したが, いずれの plot にもよくフィットした。

3. 喀痰から分離された菌株の薬剤感受性成績

Table 3 に各喀痰から分離された P. aeruginosa の各抗菌薬に対する薬剤感受性試験の成績を示す。I 株を除く菌株の imipenem に対する MIC は 4 μg/ml 以上の値を示した。B 株, C 株および F 株の aztreonam に対する MIC 値はそれぞれ, 4 μg/ml, 8 μg/ml, 2 μg/ml であったが他の菌株は 16 μg/ml 以上の値を示した。

4. 喀痰から分離された菌株の β-ラクタマーゼ活性

喀痰から分離された菌株の β-ラクタマーゼ活性を Table 4 に示した。C 株および E 株の V_{max} 値がそれぞれ 0.495 μM/sec, 0.673 μM/sec と高い値を示した。一方, A 株および F 株から抽出した β-ラクタマーゼ活性は各々 0.012 μM/sec, 0.011 μM/sec と低い値を示した。

III. 考　　　察

現在まで, 喀痰中 β-ラクタマーゼ活性の測定は, あらかじめ酵素と基質を反応させその分解産物を高速液体クロマトグラフィーで定量して求める方法が報告されている[1]。しかし, この方法は検体の処理がきわめて煩雑であること, 迅速に検査が行えないこと, 酵素学的パラ

Table 1. β-lactamase activity measurements: methods and conditions

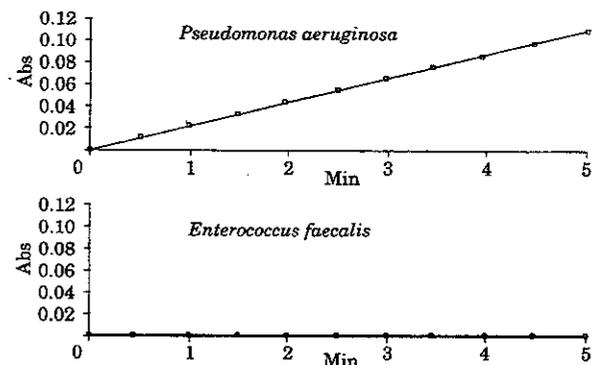| Substrate	Nitrocefin
Substrate conc	10, 25, 100 μM
Measuring wave length	482 nm
Measuring equipment	Absorption photometer (Beckman DU 640)
Reaction time	1 min
Reaction temp	37℃

Fig. 3. Time course of absorbance changes in reaction of nitrocefin and extracts of sputum in which *Pseudomonas aeruginosa* or *Enterococcus faecalis* were detected.

Table 2.　β-lactamase in sputum: enzymologic parameters

Specimen	Bacterial count (CFU/ml)	V_{max} (μM/sec)	K_m (μM)	V_{max}/K_m
A	10^5	0.132	37.3	0.0035
B	10^7	0.014	23.1	0.0006
C	10^7	0.071	39.2	0.0018
D	10^7	0.093	37.6	0.0025
E	10^6	0.165	33.7	0.0049
F	10^7	0.023	25.2	0.0009
G	10^6	0.059	24.1	0.0024
H	10^5	0.059	22.3	0.0026
I	10^6	0.011	17.4	0.0006
J	10^7	0.005	127.5	<0.0001

Table 3.　Drug sensitivity of isolated strains to various β-lactam anitibiotics

Drug	MIC(μg/ml)								
	A	B	C	D	E	F	G	H	I
Piperacillin	16	8	8	16	128	4	64	>128	4
Imipenem	64	4	16	32	64	16	32	64	2
Cefsulodin	4	16	16	4	64	2	4	64	16
Cefoperazone	64	16	32	16	64	16	16	>128	16
Ceftazidime	16	4	4	4	16	2	8	64	4
Cefepime	16	2	4	4	16	2	8	64	4
Aztreonam	16	4	8	16	16	2	16	64	16

Table 4.　β-lactamase extracted from isolated strains: enzymologic parameters

Specimen	V_{max} (μM/sec)	K_m (μM)	V_{max}/K_m
A	0.012	18.0	0.001
B	0.114	29.3	0.004
C	0.495	42.7	0.012
D	0.150	30.9	0.005
E	0.763	66.8	0.011
F	0.011	16.0	0.001
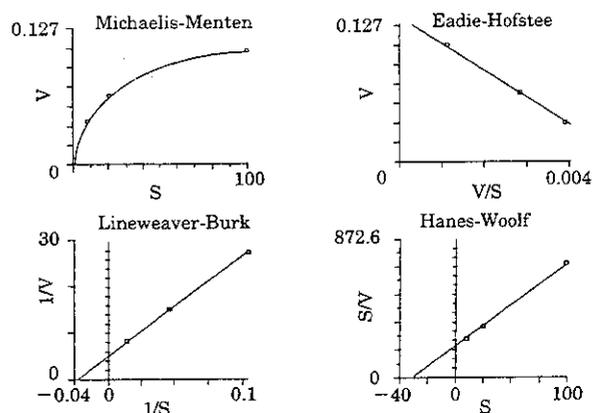
Fig. 4.　Various plottings observed while calculating enzymologic parameters.

メータを算出することが困難なことなどの理由から一般的な応用には限界があると考えられる。

今回検討したβ-ラクタマーゼを検出する方法は，簡便，迅速かつ高感度な方法であった。さらに，酵素学的パラメータを算出することも可能であり，V_{max}およびK_m値に対する解析を加えればβ-ラクタマーゼの型の推定も可能であるものと考えられた。しかし，現在問題となっている基質特異性拡張型β-ラクタマーゼ[5]や複数の型のβ-ラクタマーゼが一つの菌体内に存在する場合[6]などのデータの解析は今後の課題である。しかし，β-ラクタマーゼ阻害剤を併用し，酵素学的パラメータの詳細な解析を加えれば，喀痰中に存在するβ-ラクタマーゼの型もある程度判別することが可能となるものと考えられる。

喀痰中β-ラクタマーゼの活性と分離された菌種のMICとの間にいくつか食い違う結果が認められた。この結果が乖離した原因として，β-ラクタマーゼ以外の要因が薬剤感受性に影響していることの他に，MIC測定をした菌株が，喀痰中に存在する多数の菌株の中の一部の菌株であることが挙げられる。すなわち，測定した菌株のMIC値は，喀痰中のβ-ラクタマーゼ活性が，

喀痰中に存在するすべての菌株のβ-ラクタマーゼ活性を表わすものではないと考えられる。

一方，今回報告した方法の問題点としては，検体を採取した時点で抗菌薬が投与されている場合が挙げられる。すなわち，喀痰中のβ-ラクタマーゼと投与されたβ-ラクタム系抗菌薬が結合している場合，正確な酵素学的パラメータを得ることはできないと考えられる。したがって，この方法を実施する場合に重要なことは，患者に投与されている抗菌薬の情報を得ることである。さらに，喀痰の塗抹検鏡の情報を加味することにより起炎菌の推定にも役立つと考えられた。

今後は喀痰以外の検体中に存在するβ-ラクタマーゼの活性測定に関する検討を加えるとともに，さまざまな型のβ-ラクタマーゼの酵素学的パラメータに関するデータを収集し，更なる応用の可能性について検討を加える予定である。

謝　辞

より実施した。

文　献

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Novel method to determine β–lactamase activity in sputum

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In order to detect β–lactamases in sputum directly, we developed a method to determine β–lactamase activity for *Pseudomonas aeruginosa* isolated from sputum specimens. The extraction procedures from sputum were carried out within 30 minutes. A satisfactory linear relationship was obtained when extracts from sputum containing *P. aeruginosa* (10^6cfu/ml) were added to 100μM nitrocefin solution and absorbance was measured for 5 minutes at 37℃. On the other hand, β–lactamase activity was not identified with samples of *Enterococcus faecalis* (10^5cfu/ml). Subsequently, we examined the detection limit using samples with different bacterial quantities and found as a result that β–lactamase activity could be detected with samples of *P. aeruginosa* of 10^6cfu/ml and above. In addition, the K_m value of sputum containing penicillinase–producing *Escherichia coli* (10^7cfu/ml) was as high as 127μM and significantly differed from those values for cephalosporinase–producing *P. aeruginosa*. These results suggested that this newly developed method was able to detect β–lactamase activity in sputum samples quickly, conveniently and sensitively.